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Research Article

Testosterone-dependence of oxidative stress in Sprague-Dawley rats fed a high salt diet

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ABSTRACT

Background: One of the mechanisms by which high salt diet (HSD) induces hypertension is by increasing the generation of reactive oxygen species (ROS). Salt sensitivity exhibits sex difference which is higher in males when compared with females. Likewise, sex disparity in oxidative stress has also been suggested. However, the role of the sex steroids in these sex differences is not clear. Therefore, experiments were designed to assess the role of testosterone on the oxidative status of male rats fed a high salt diet. **Methods:** Weanling male Sprague-Dawley rats were either sham-operated or orchidectomised under (90mg/kg bodyweight ketamine and 10mg/kg bodyweight xylazine i.p) anesthesia, with or without testosterone replacement (10mg/kg sustanon 250® i.m) once in 3 weeks. They were placed on a diet with normal 0.3% or high 8% NaCl content for 6 weeks. Blood pressure (BP) was measured via arterial cannulation. Levels of lipid peroxidation (LP), superoxide dismutase (SOD), bilirubin and testosterone were measured in the serum. **Results:** Orchidectomy attenuated the BP elevating effect of a high salt diet, but concomitant replacement of testosterone in orchdectomised rats restored the BP values towards that observed in sham-orchidectomised animal. Orchidectomy abolished while testosterone replacement re-established the significant increase ($p<0.001$) in LP of the HSD group when compared with the control. High salt diet significantly reduced ($p<0.01$) the serum levels of SOD. Orchidectomy prevented the reduction in SOD level effect of a high salt diet but testosterone replacement re-established it. Findings from this study show that orchidectomy reduced oxidative stress in male Sprague-Dawley rats fed a high salt diet suggesting a role for testosterone in ROS generating effect of a high salt diet.

INTRODUCTION

Excess salt in the diet causes hypertension in both human (Bursztyn *et al.*, 2013) and animal (Carlström *et al.*, 2007). One of the mechanisms by which a high salt diet induces hypertension includes generation of reactive oxygen species and its attending elevation of oxidative stress (Huang *et al.*, 2016; Dornas *et al.*, 2017). Several studies have reported the reactive oxygen species generating effect of a high salt diet (Tian *et* al., 2007; Ge *et* al., 2017; Huang *et* al., 2017). Bilirubin, apart from being an end-product of *haem* catabolism, is also an endogenous antioxidant (Stocker *et al.*, 1987; Vitek, 2017). Some studies have demonstrated an inverse relationship between serum bilirubin and oxidative stress-mediated diseases: including coronary artery disease (Wiesel *et al.*, 2001; Gupta *et al.*, 2016); atherosclerotic diseases (Vitek, 2017), angiotensin II-mediated hypertension (Kitiyakara *et al.*, 1998); and *in vivo* renal ischemia-reperfusion injury (Adin *et al.*, 2005; Kirkby *et al.*, 2006). Likewise, serum level of bilirubin has been shown to have an inverse relationship with cardiovascular diseases (Kunutsor *et al.*, 2015; Suh *et al.*, 2018).

High salt diet has also been shown to elicit some other deleterious effects on different organs of the body besides elevation of blood pressure. For example, a high salt diet causes cardiac, renal and vascular hypertrophy and dysfunction with or without concomitant elevation in blood pressure (de Wardener *et al.*, 2002; Huang *et al.*, 2016; 2017). Sensitivity to a high salt diet is sex dependent, its adverse effect is usually more pronounced in males when compared with females in human (Bursztyn *et al.*, 2013). Likewise, a high salt diet elevated blood pressure to a greater extent in male when compared with female rats (Hinojosa-

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Laborde *et al.*, 2004). In the same vein, some clinical studies have shown that oxidative stress is higher in men than women (Ide *et al.*, 2002), a finding that is consistent with reported higher blood pressure in males compared to females (Bursztyn *et al.*, 2013; Maranon *et al.*, 2013; Choi *et al.*, 2017; Colafella *et al.*, 2018;). Several experimental studies have also shown oxidative stress to be higher in male animals compared to female, for example, angiotensin II infusion in male rats resulted in an increase in blood pressure with a corresponding increase in superoxide production in an isolated aorta (Rajagopalan *et al.*, 1996), while angiotensin II infusion in female rats, though elevates blood pressure, failed to change the activity of NADPH oxidase (Ebrahimian *et al.*, 2007), or the expression of $p67^{phox}$, a subunit of NADPH oxidase (Tatchum-Talom *et al.*, 2005). Likewise, tempol a superoxide dismutase mimetic reduced blood pressure in male spontaneously hypertensive rat (SHR) while it had no effect on the blood pressure of female SHR (Yanes *et al.*, 2005; Sartori-Valinotti *et al.*, 2007) and female mREN2 Lewis rats (Chappell *et al.*, 2008). Likewise, Sex differences in haem oxygenase (HO) activity have also been suggested. For instance, the effect of bilirubin on the development of hypertension was reported to be more evident in females than male human subjects (Chin *et al.*, 2009). Likewise, trauma and haemorrhage were reported to double the hepatic HO-1 expression in female rats compared with male rats (Toth *et al.*, 2003), implicating sexual differences in the activity of bilirubin. However, there is paucity of information on the level of bilirubin, and the effect of androgen on level of bilirubin in salt-induced hypertension. The role of androgens in ROS generating and antioxidant enzyme depleting effects of a high salt diet is also not clear. Therefore, considering the sexual differences in response to a high salt diet, and the implication of sex steroids in this effect, we choose to investigate the effect of testosterone on oxidative stress in male Sprague-Dawley rats fed a high salt diet.

MATERIALS AND METHODS

Thirty-six 8 weeks old weanling male Sprague Dawley rats weighing between 90-110g were obtained from the Department of Laboratory Animal Sciences, (DLAS), BioMedical and Technology (BMT) wing Sree Chitra Tirunal Institute for Medical Sciences and Technology (SCTIMST) Kerala, India. They were housed in steel cages and maintained under standard lighting conditions 12 hours light, 12hours dark period. Food and water were provided *ad libitum*. The rats were divided into six groups of six rats each. Groups 1 and II were sham orchidectomised rats, groups III and IV were orchidectomised rats, groups V and VI were rats that were given Sustanon® injection as testosterone replacement following orchidectomy. For orchidectomy, rats were anesthetized with ketamine and xylazine (90mg and 10mg/Kg/body weight i.m) (Oloyo *et al.*, 2011), respectively, for bilateral removal of the testes under aseptic surgical conditions while in groups I and II rats, the scrotal sacs were opened and sutured back as a model of sham-orchidectomy. All operated rats received an injection of penicillin 300,000 i.u/Kg body weight at the time of surgery to prevent infections and were allowed a 3-day recovery period before the beginning of the experiments (Oloyo *et al.*, 2011). After recovery from anaesthesia, all animals were returned to their cages. Rats in groups I, III, and V were fed with rat diet containing normal salt (NS) concentration (0.3% NaCl) and tap water for 6 weeks. Rats in groups II, IV and VI were fed with a high salt (HS) diet (8% NaCl), and tap water *ad libitum*, for 6 weeks. Group V and VI rats received 10mg/Kg body weight of testosterone suspension (Sustanon 250® i.m) once in 3 weeks during the study, for testosterone replacement (Oloyo *et al.*, 2011). Sustanon 250® is a trade name for an oil-based injectable blend of four esterized testosterone compounds viz: 30mg Testosterone Propionate, 60mg Testosterone Phenylpropionate, 60mg Testosterone Isocaproate, and 100mg Testosterone Decanoate. The composition is a blend of fast, short acting and slow but long acting testosterone esters. Therefore, group I = Sham plus normal salt (Sham + NS); group II = Sham plus High Salt (Sham + HS); group III = Orchidectomy plus normal salt (Orch + NS); group IV = Orchidectomy plus normal salt (Orch + HS); group V = Orchidectomy plus testosterone plus normal salt (Orch + Tes + NS) and group VI = Orchidectomy plus testosterone plus high salt (Orch + Tes + HS).

Determination of Body Weight

The animals were weighed before and after the six - week experimental period, using a Duet top loading weighing scale (Salter, England). At the end of the experimental period, the percentage weight gain of the animals was calculated as differences between the final body weight and initial body weight divided by initial body weigh multiply by 100.

$$\frac{\text{Final body weight} - \text{Initial body weight}}{\text{Initial body weight}} \times 100$$

The percent changes in body weight were compared across the groups.

Determination of Heart and Kidney weight indices
After sacrifice the heart and kidneys were removed, carefully cleared of connective tissues, dried between filter paper and weighed. The weight index of each organ was taken as the division of such organ by the total body weight multiplied by 100. And the weight index of each organ was compared across the groups.

Measurement of Blood Pressure in the Animals
At the end of the experimental period, blood pressure was measured via non-invasive tail plethysmography method as described previously (Oloyo *et al*, 2013). Briefly, the conscious rats were placed in a restrainer on a heated pad (37°C) and allowed to adapt/rest inside for 15 min before blood pressure was measured. The rat tail was placed inside a 9 mm or 11 mm tail cuff, and the cuff was inflated and released several times to allow the animal to become conditioned to the procedure. Five consecutive blood pressure and heart rates measurements were obtained using the non-invasive blood pressure monitor MP35 (BIOPAC System Inc., Goleta, California, USA), which was connected to a computer. Blood pressure tracings were obtained through preinstalled software for BSL Pro.3.7.

Determination of Lipid peroxidation in the Serum
Lipid peroxidation was measured by determining malondialdehyde (MDA) production, using thiobarbituric acid reactive substances (TBARS) assay. The methods of Niehaus and Samuelson (1968), modified by Yagi (1998) were used. Serum from blood collected over ice was used for the experiment. Briefly, trichloroacetic acid (TCA) – Thiobarbituric acid (TBA) reagent was prepared as follows; 3g of TCA was weighed in brown bottle and was dissolved in 9.58ml 0f distilled water to which 0.42ml of concentrated HCL was added. 75mg of TBA was weighed and dissolved in 10ml distilled water. The two separate solution were then added together to form the TCA – TBA reagent. Lipid peroxides were measured by adding 2ml of TCA - TBA reagent to 1ml of the sera. The mixture was then heated in a boiling water bath for 15 minutes. TBA and MDA reacts to form a Schiff base adducts under high temperature and acidic condition to produce a chromogenic product that can be easily measured using analytical techniques such as spectrophotometry (Lapenna *et al*, 2001). After cooling, the suspension was centrifuged at 1000G for 10 minutes. The supernatant was then separated and absorbance was measured at 532nm in a UV I Spectrophotometer (Thermo Electron Corporation, USA). The MDA concentration of the sample was calculated using the formula below:

$$\text{MDA (mmol/L)} = \frac{OD \times V \times DF}{\Sigma \quad v}$$

OD = Absorbance (Optical Density) of sample
Σ = Molar extinction coefficient = 1.56×10^5
V = Total volume of the reacting sample
v = Volume of the sample
DF = Degree of Freedom.

Serum Level of Super Oxide Dismutase (SOD)
Super oxide dismutase (SOD) level in the serum was assayed at 30°C according to the method described by Misra and Fridovich (1972). The assay was performed in 3ml of 50mM Na_2Co_3 buffer (pH 10.2), 0.02ml of the sample was added to 3ml of buffer and 0.03ml of the substrate which is epinephrine. The oxidation of epinephrine was followed in terms of the production of adrenochrome, which exhibits an absorption maximum at 480nm, with an extinction coefficient of 4020 M^{-1} cm^{-1}. A blank was prepared as follows; 0.02ml of distilled water was added to 3ml of the buffer and 0.03ml of the substrate. Absorbance of the sample was taken at 480nm over 3 to 5 minutes using a UV 1 spectrophotometer (Thermo Electron Corporation, USA). One unit of SOD was defined as the amount of enzyme, which causes 50% inhibition of epinephrine auto-oxidation. SOD concentration in the sample was calculated as follows:

$$\text{SOD (µg/ml)} = \frac{\Delta A_{480} \times V \times DF}{\Sigma \quad v}$$

ΔA_{480} = Difference in absorbance between the sample and the blank
Σ = Extinction coefficient ($4020 M^{-1} cm^{-1}$)
V = Total volume of the reacting sample
v = Volume of the sample
DF = Degree of Freedom

Serum Level of Bilirubin
Bilirubin level was measured using the Erba Chem – 7 automated machine with specific Erba reagents and assay kits (Erba, Germany) for bilirubin. The machine was calibrated with serum-based XL multical calibrator and the XL results were calculated automatically by the machine. To ensure adequate quality control normal and abnormal controls with assayed values were ran as unknown samples. Total and direct bilirubin levels determination procedure follows the Walter and Gerard (1970) method. Bilirubin is coupled with diazotized sulfanilic acid in the presence of ethylene glycol and dimethylsulfoxide (DMSO) as solvents to produce an intensely coloured diazo compound. The intensity of colour of this solution is proportional to the

concentration of the bilirubin total in the sample. In the absence of an accelerator, only conjugated (direct) bilirubin reacts. In the presence of an accelerator, DMSO, the non – conjugated bilirubin also participate in the reaction, thus determine the level of total bilirubin. Optical Density (OD) for total bilirubin reagents and standard were 0.0020 and 0.2224 respectively. The machine presents the value/concentration of total bilirubin in mg/dl. Total bilirubin assay reagent composition is as follows: Sulphalinic acid (25.6mmol/L), HCl (40mmol/L) and sodium nitrite (1mmol/L). The machine presents the value/concentration of direct bilirubin in mg/dl. Direct bilirubin assay kit's compositions are sulphanilic acid (27.74 mmol/L), HCl (40 mmol/L), and sodium nitrite (1.38 mmol/L).

Measurement of serum concentration of testosterone

Concentration of testosterone in the serum was determined as previously described (Oloyo *et al*, 2013). Briefly, whole blood was collected via cardiac puncture using a 5 ml syringe and 21-gauge needle and serum was separated and stored at −80°C. Serum testosterone levels were measured by enzyme-linked immunoassay method using a commercial kit from Biotech Laboratories (Suffolk, UK) according to the manufacturer instructions. The kit uses the principle of competitive microplate enzyme immunoassay, whereby testosterone that is present in the sample competes with enzyme–testosterone conjugate for binding with an anti-testosterone-coated microplate to form an antigen–antibody complex.

Statistical Analysis

The collected data were expressed as mean ± S.E.M and analysed using one-way analysis of variance (ANOVA). Student-Newman-keuls post Hoc test was used to identify differences between individual means. Confidence interval was placed at 95%, so that in all cases a value of $P < 0.05$ was considered significant.

RESULTS

Body weight

Table 1 shows the per cent change in the body weight of the animal after the six-week experimental period. At the end of the six weeks feeding period, there was an increase in the body weights of all the rats across the groups. However, a high salt diet significantly reduced the per cent increase in the body weight when compared with that of the group fed a normal diet, but orchidectomy abolished the difference that exists in the orchidectomised Sprague-Dawley rats fed a high salt diet in the presence or absence of testosterone replacement.

Table 1. Percent change in body weight, cardiac and renal weight indices and systolic blood pressure

Groupings (n = 6)	weight gain (%)	Heart weight index	Kidney weight index	Systolic Pressure (mmHg)
Sham + NS	58.73 ± 0.33	0.28 ± 0.006	0.57 ± 0.05	130 ± 3
Sham + HS	46.71 ± 1.04*	0.33 ± 0.007*	0.65 ±0.04*	150 ± 4*
ORCH + NS	57.71 ± 0.80	0.28 ± 0.008	0.55 ±0.03	120 ± 4#
ORCH + HS	51.10 ± 0.49	†0.29 ± 0.005	†0.57 ±0.02	†134 ± 5*
ORCH + TES + NS	58.79 ± 0.55	0.29 ± 0.005	0.55 ±0.01	132 ± 3
ORCH + TES + HS	50.57 ± 0.47*	0.34 ± 0.005*	0.67 ± 0.02*	144 ± 3*

Data are expressed as mean ± S.E.M. (n = 6). Significant increase (*p<0.05) when compared with their corresponding control groups. Significant decrease (#p<0.05) when compared with sham plus normal salt group. Significant decrease (p<0.05) when compared with the corresponding control groups. †significant decrease (p<0.05) when compared with sham plus high salt group.

per cent increase in body weight between rats in the normal salt and high salt groups while testosterone replacement restored the difference that exists in the per cent body weight gain of the normal and high salt fed rats.

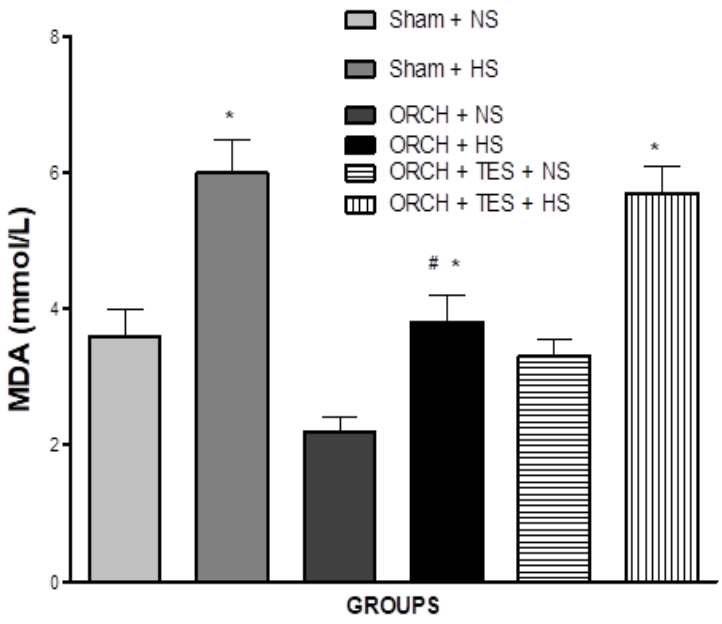

Fig. 1. Lipid peroxidation level in the serum of the rats across the groups n = 6. Data expressed as mean ± S.E.M. Significant increase (*p<0.05) when compared with their corresponding control groups. Significant decrease (#p<0.05) when compared with sham and /or testosterone replacement groups that are placed on a high salt diet.

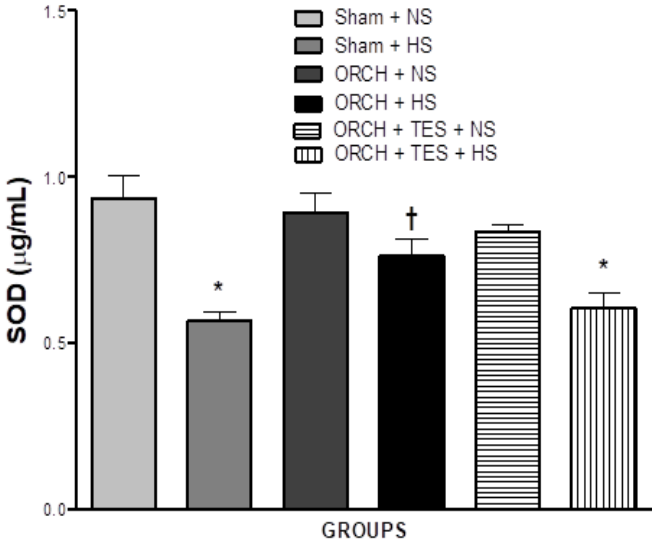

Fig. 2. Serum levels of super oxide dismutase (SOD) in the rats across the groups. Data expressed as mean ± S.E.M. Significant decrease (*p<0.05) when compared with the corresponding control groups. Significant increase (†p<0.05) when compared with sham and / or testosterone replacement group that were placed on a high salt diet.

Organ weights

The heart and kidney weight indices are presented in table 1. The relative weights of the heart and the kidney to the body weights were obtained and recorded as weight indices. There was a significant increase ($p < 0.05$) in the mean heart weight index of the sham plus high salt diet group when compared with that of the sham plus normal salt diet group (control). However, there was a significant decrease ($P < 0.05$) in the heart weight index of orchidectomy plus high salt group when compared with sham plus high salt diet group, while there was no significant difference in the heart weight index between the orchidectomised and high salt group when compared with the sham and normal salt group (control). This suggests that orchidectomy prevented cardiac hypertrophic effect of a high salt diet as heart weight indices in orchidectomised plus high salt diet rats are similar to that of the control rats.

There was a significant increase ($p < 0.05$) in the kidney weight index of high salt diet group when compared with their corresponding control groups, except in the orchidectomised groups where there was no significant difference between the kidney weight index of the normal diet group and high salt group. There was a significant decrease ($P < 0.05$) in the kidney weight index of orchidectomy and high salt group when compared with that of the sham and high salt diet group (Table 1).

Blood Pressure parameter

Table 1 shows the systolic blood pressure (SBP) of the rats across the groups. At the end of the 6-week experimental period, a high-salt diet significantly increased ($p < 0.05$) the SBP of the rats compared with those fed a normal-salt diet. Orchidectomy reversed the blood pressure elevating effect of a high salt diet as the SBP in the orchidectomy plus high-salt diet group was significantly less ($p < 0.05$) compared with the sham plus high-salt diet group. There was also no significant difference in the SBP of orchidectomy plus high salt diet when compared with that of the Sham plus normal salt diet, suggesting that orchidectomy restored BP towards normal in rats fed a high salt diet. However, SBP was significantly lower in orchidectomy plus normal salt rats when compared with that of sham plus normal salt rats (control). Conversely, testosterone replacement almost restored SBP to the level observed in sham orchidectomised animals as the increase in SBP of rats in the testosterone replacement groups was significantly higher ($p < 0.05$) compared with their corresponding orchidectomised groups without testosterone replacement and there were no significant differences in SBP of rats with testosterone replacements when compared with their corresponding sham orchidectomised groups i.e. (Sham + NS vs. Orch + Tes + NS and Sham + HS vs. Orch + Tes + HS).

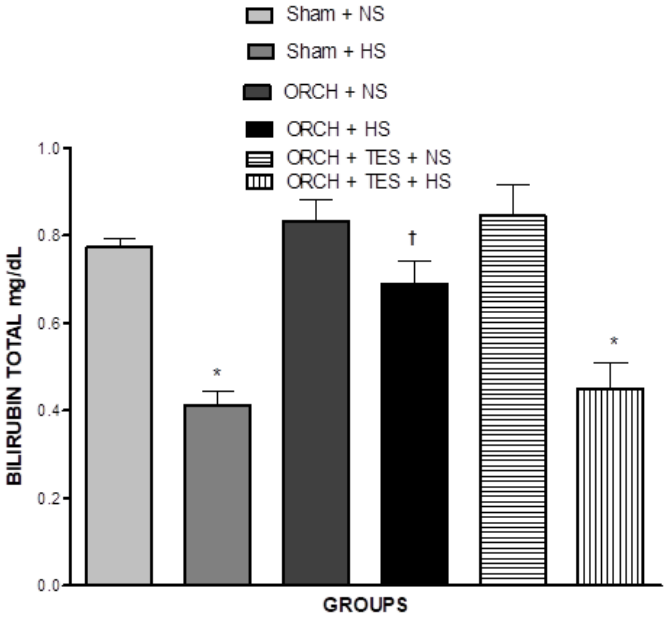

Fig. 3. Serum level of bilirubin total in the rats across the groups. n = 6. Data are expressed as mean ± S.E.M. Significant decrease (*p<0.05) when compared with the corresponding control groups. Significant increase (†p<0.05) when compared with both sham and/ or testosterone replacement group placed on a high salt group.

Level of Lipid Peroxidation in the Serum

Figure 1 shows the level of lipid peroxidation in the serum of the rats across the groups. There was a significant increase ($P < 0.05$) in the level of lipid peroxidation in the serum of the high salt diet groups when compared with their corresponding normal salt groups. However, there was a significant decrease ($P < 0.05$) in the lipid peroxidation level in the serum of the orchidectomy plus high salt group when compared with the sham plus high salt diet group. On the other hand, testosterone replacement significantly increased ($P < 0.05$) the lipid peroxidation level in the serum towards that in the sham groups, as there was no significant difference between serum lipid peroxidation in testosterone replacement group when compared with the sham groups.

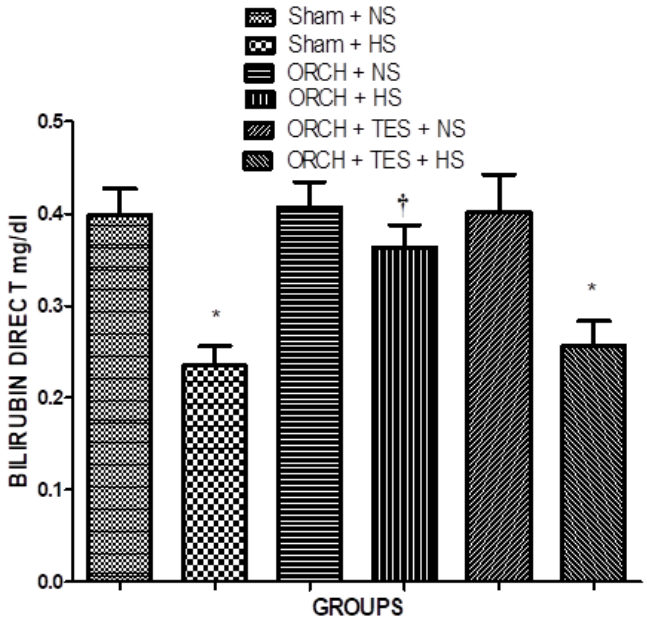

Fig. 4. Serum level of bilirubin direct in the rats across the groups. n = 6. Data are expressed as mean ± S.E.M. Significant decrease (*$p<0.05$) when compared with the control group. †Significant increase (†$p<0.05$) when compared with sham and/ or testosterone replacement group placed on a high salt group.

Super Oxide Dismutase (SOD) level in the serum

Figure 3 shows the level of SOD in the serum of the rats across the groups. There was a significant decrease ($P < 0.05$) in the SOD level in the serum of the sham plus high salt diet group when compared with the sham plus normal salt diet group. The difference in the serum level of SOD between high salt fed and normal salt fed rats was abolished by orchidectomy. However, there was a significant increase ($P < 0.05$) in the serum level of SOD in the orchidectomy plus high salt group when compared with the sham plus high salt diet group, and testosterone replacement re-established the difference ($p<0.05$) in the level of SOD in the sera of high salt and normal salt groups.

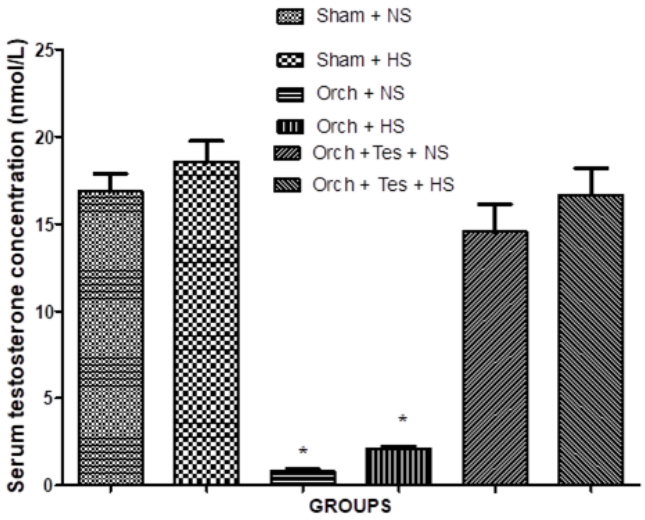

Fig. 5. Serum level of testosterone in the rats across the groups. n = 6. Data are expressed as mean ± S.E.M. Significant increase (*$p<0.05$) when compared with the corresponding sham and/ or testosterone replacement group.

Serum level of Total and direct Bilirubin

Figures 4 and 5 show the level of total and direct bilirubin in the serum of the rats across the groups. There was a significant decrease ($P < 0.05$) in the serum level of bilirubin in the high salt diet groups when compared with their corresponding control groups. However, there was a significant increase ($P < 0.05$) in the serum level of bilirubin in orchidectomy and high salt diet group when compared with the sham plus high salt diet group. Replacement of testosterone in the orchidectomised rats restored the serum levels of bilirubin towards that found in the sham - orchidectomised rats.

Serum concentration of testosterone

Figure 6 shows the serum level of testosterone across the groups of rats. Orchidectomy significantly reduced ($p < 0.001$) the level of testosterone in the serum compared with the sham - orchidectomised and testosterone replacement groups. Testosterone replacement restored the serum testosterone level to that observed in the sham orchidectomised groups as there was no significant difference ($p > 0.05$) between the testosterone replacements and the sham – orchidectomised groups. This indicates successful methods of testosterone withdrawal and replacement in the present study.

DISCUSSION

Results from this study show that orchidectomy reduced but testosterone replacement re-established the oxidative stress effects of a high salt diet in male Sprague-Dawley rats. The increase in the heart and kidney weight indices of rats fed a high salt diet is consistent with the increased oxidative stress in this same group of rats in this study. The cardiac and renal hypertrophy as well as oxidative effects of a high salt diet is also consistent with the findings of others. For instance, it has been recognized that excess salt is strongly associated with cardiac hypertrophy, a structural pattern observed in both hypertensive men and rats independently of the level of blood pressure (Huang *et al*, 2016, Imaizumi *et al.*, 2016). Likewise, a temporal link between increased NaCl intake and aortic hypertrophy has also been noted (Limas *et al.*, 1980; Partovian *et al.*, 1998) in spontaneously hypertensive rats (SHR) in the absence of a significant change in blood pressure. However, those studies did not consider the effect of androgen on the hypertrophic effect of a high salt diet. Findings from the present study implicate testosterone in the link between cardiac and renal hypertrophy and ROS generating effect of a high salt diet in male rats.

Lipid peroxidation is a major indicator of oxidative stress (Lef'evre *et al.*, 1998, Ayala *et al.*, 2014) and corresponds with the concentration of superoxide radicals in a tissue. The level of lipid peroxidation in the serum was measured as an indication of the level of oxidative stress in the rats. Significant increase in lipid peroxidation in the serum of the high salt diet groups suggests an increase in oxidative stress in rats in these groups. This finding is consistent with other studies that report increasing oxidative stress effect of a high salt diet (Huang *et al.*, 2016; Tian *et a.l*, 2007). ROS activities in other organ systems, such as the heart, nervous system, and kidneys, have also been implicated in the pathophysiology of hypertension (Imaizumi *et al.*, 2016; Huang *et al.*, 2016; 2017). In particular, increased renal O_2^- production is associated with NO bio-inactivation, which influences afferent arteriolar tone, tubuloglomerular feedback responses, and sodium reabsorption, which are important in long-term BP regulation (Wilcox, 2002). High salt diet has been reported to affect both cardiac and renal functions negatively (Huang *et al.*, 2016; 2017). It could be that the negative impact of a high salt diet on the heart and kidney is mediated through its ROS generating effect. Orchidectomy attenuated the increase in lipid peroxidation, as observed in the orchidectomised and high salt diet group, while testosterone replacement following orchidectomy increased the lipid peroxidation level almost back to what is obtained in the sham groups. This result implicates testosterone in the oxidative stress promoting effect of a high salt diet. This current result is consistent with that obtained from the cardiac and renal weight indices experiment. The significant increase in the cardiac and renal weight indices of the high salt fed rats could be due to the increased oxidative stress observed in the serum of rat from this group because increased ROS generation has been implicated in cardiac and renal hypertrophy (Huang *et al.*, 2016; 2017). Likewise, the finding that orchidectomy reduced the cardiac and renal hypertrophic effect of a high salt diet is also consistent with the finding that orchidectomy attenuated the ROS generating effect of a high salt diet in this study.

Superoxide dismutase (SOD) is one of the most important antioxidant enzymes in the body (Berry *et al.*, 2001). Maintaining a balance between ROS generation and antioxidant system in the body is necessary in preventing oxidative stress and its consequential negative effect. The serum level of SOD was measured as an indicator of the antioxidant system in the body. The decrease in the level of SOD from serum of rats fed a high salt diet suggests the depletion of this important endogenous antioxidant system in this group of rats. This finding is consistent with the elevated level of lipid peroxidation in the serum of the rats fed a high salt diet. It implies that the available SOD is used up in quenching the generated ROS by a high salt diet as suggested by the elevated level of lipid peroxidation. This is consistent with the data on lipid peroxidation discussed above. An increase in the level of lipid peroxidation in the body indicates an increase in ROS generation e.g. O_2^- which is a substrate for SOD. SOD reacts with O_2^- converting it to hydrogen peroxide (H_2O_2) and H_2O_2 in another step reaction is converted to water and molecular oxygen (Berry *et al.*, 2001). The significant increase in SOD level of the orchidectomy plus high salt diet group when compared with sham plus high salt diet group suggests orchidectomy counteracted ROS generating effect of high salt diet therefore, reducing the usage of antioxidant system (SOD) or it increased the production of SOD in the body as a way of preventing oxidative stress. The implication of testosterone in increased oxidative stress in animals fed a high salt diet is reinforced by elevation of lipid peroxidation and concomitant reduction in SOD level in testosterone replacement groups. These findings are indicative of testosterone – dependence of oxidative stress elevating effect of a high salt diet in the rat.

The result of the present experiment indicates that high salt diet decreased the total serum bilirubin levels.

Bilirubin is not merely an end product of haem degradation but a potent endogenous antioxidant which can be destroyed by ROS (Stocker *et al.*, 1987; Vitek, 2017). Bilirubin usually acts by inhibition of NADPH oxidase (Lanone *et al.*, 2005) and of protein kinase C activity (Sano *et al.*, 1985; Amit *et al.*, 1993). The reduction in the serum bilirubin level in the high salt diet group could be as result of an increase in the ROS level in these groups of rats. An increase in the ROS level will consequently lead to a decrease in the level of antioxidant system such as bilirubin, as the latter is used to mop up the excess ROS. Some studies have reported a relationship between serum bilirubin and oxidative stress-mediated diseases, including coronary artery disease (Endler *et al.*, 2003; Novotny *et al.*, 2003), angiotensin II-mediated hypertension (Pflueger *et al.*, 2005), and renal ischemia-reperfusion injury (Adin *et al.*, 2005; Kirkby *et al.*, 2006). High salt diet generates ROS that consume bilirubin, this possibly might be the reason for the reduced serum level of bilirubin observed in rats fed a high salt diet in this study. Reduction in the serum bilirubin level observed in rats fed a high salt diet was attenuated by orchidectomy, while testosterone replacement re-established it. This finding implicates testosterone in the antioxidant activities of bilirubin. Although it is not imminently clear how testosterone reduces concentration of serum bilirubin, but sex disparity in the haem oxygenase activity, which is the rate-limiting enzyme to produce bilirubin has been reported. For instance, Toth *et al.*, (2003) reported that trauma and haemorrhage doubled the hepatic HO-1 expression, in female rats compared with male rats. Likewise Chin *et al.*, (2009) reported that subjects with higher bilirubin level showed a lower incidence of hypertension than did the subjects with lower bilirubin level, especially in females. Novotny and Vitek, (2003) reported that in humans, mildly increased serum bilirubin levels is a decreased risk for the development of coronary artery disease and atherosclerosis. Likewise, in hyperbiliruminaemic Gunn rats infused with angiotensin II, the rise in systolic blood pressure was markedly blunted, and oxidative stress was attenuated when compared with control (Pflueger *et al.*, 2005). The finding of the present study agrees with the above reports. In this study, an observation worthy of note is the lower blood pressure parameters in groups with higher serum level of bilirubin. Blood pressure reducing effect of orchidectomy is consistent with its serum bilirubin elevating effect in rats fed a high salt diet. Therefore, increasing serum bilirubin and SOD levels, either by promoting their production or preventing excess ROS generation which could have reduce the bilirubin and SOD bioavailability could be one of the mechanisms by which orchidectomy prevents or attenuates blood pressure elevation in rats fed a high salt diet. On the other hand, blood pressure elevating effect of testosterone could be partly mediated by decreased serum bilirubin and SOD, which increases oxidative stress and consequently promotes endothelial dysfunction.

In conclusion, testosterone potentiates the cardiac and renal hypertrophic as well as oxidative stress effect of a high salt diet, and these mechanisms appear to underlie the sexual differences in response to salt stress.

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